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Development in the Absence of Skeletal Muscle Results in the Sequential Ablation of Motor Neurons from the Spinal Cord to the Brain

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Mice lacking the transcription factors *Myf-5* and *MyoD* lack all skeletal muscle and therefore present a unique opportunity to investigate the dependence of nervous system development on myogenesis. Motor neurons arose normally in the spinal cord of mutant embryos and by birth all somatic motor neurons were eliminated by apoptosis. By contrast, interneurons were not affected. Proprioceptive sensory neurons in the dorsal root ganglia underwent apoptosis. The facial motor nucleus was ablated of motor neurons and contained large numbers of apoptotic bodies. Surprisingly, giant pyramidal neurons were absent in the motor cortex without any corresponding evidence of apoptosis. The epaxial and cutaneous component of dorsal ramus failed to form in the absence of the myotome. Therefore, we conclude that nervous development is more intimately coupled to skeletal myogenesis than has previously been understood.

Key Words: **motor neuron; type Ia afferent; pyramidal cell; apoptosis; *Myf-5; MyoD*.**

INTRODUCTION

The determination of progenitors of motor neurons is regulated in part by signals from the notochord and floor plate of the neural tube (Yamada *et al.*, 1991, 1993). Sonic hedgehog (Shh) is expressed in the notochord and floor plate in the trunk where it functions in induction of motor neurons, as well as in patterning of the sclerotome and dermamyotome (Fan and Tessier-Lavigne, 1994; Roelink *et al.*, 1995; Roelink, 1996; Tanabe *et al.*, 1995; Jessell and Goodman, 1996). In response to these signals, the progenitors of motor neurons situated in the ventricular epithelium of the ventral neural tube migrate laterally to settle in a single continuous primary motor column (Hollyday, 1980; Ericson *et al.*, 1992, 1996). Later, neuronal migration and selective cell death are believed to further shape the developing motor columns forming of the visceral column of Terni and the median and lateral motor columns (Hollyday and Hamburger, 1977). The visceral motor column contains visceral motor neurons that indirectly innervate smooth muscle. The median motor column contains somatic motor neurons that directly project their axons to axial and body wall skeletal muscle. The lateral motor column contains somatic motor neurons that innervate limb skeletal muscle (Tanabe and Jessell, 1996).

Motor neurons become specified to innervate particular targets early in development prior to their projection of axons to their respective targets (Tosney *et al.*, 1995). The basis of this specificity may be regulated in part by a group of LIM-domain transcription factors that include Islet-1 (Isl-1), Islet-2 (Isl-2), Lim-1, Lim-2, and Lim-3 (Tsuchida *et al.*, 1994; Pfaff *et al.*, 1996). For example, all motor neurons that initially express Isl-1 followed by Isl-2 project to ventral body wall muscles, whereas motor neurons that also express Lim-1 will project to muscles in the limb (Tosney *et al.*, 1995; Tsuchida *et al.*, 1994). Presumably, the expression of different combinations of LIM proteins defines motor neuron identity and allows discrimination between the different cues that guide axonal projection. Motor neurons that innervate axial muscles project axons toward signals provided by dermamytomal myogenic precursors, whereas motor neurons that innervate limb muscles project axons toward signals provided by the limb mesenchyme (Tosney, 1987, 1988; Phelan and Hollyday, 1990).

During development of the nervous system, motor neurons are believed to compete for target sites on

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skeletal muscle with their eventual survival being contingent on trophic support from the target muscle. This hypothesis is supported by observations that removal of targets during embryogenesis results in loss of the innervating neurons (Oppenheim, 1981, 1991, 1996; Hamburger and Oppenheim, 1982). In addition, provision of specific neurotrophic factors that are expressed in muscle can rescue motor neurons from induced programmed cell death (Oppenheim *et al.*, 1991, 1992, 1995; Sendtner *et al.*, 1992a,b; Yan *et al.*, 1992, 1993, 1994, 1995; Henderson *et al.*, 1993, 1994; Koliatsos *et al.*, 1993; Oppenheim, 1996). This competition for targets during mouse development is believed to result in about a 60% loss of the motor neurons in the spinal cord (Lance-Jones, 1982; Oppenheim *et al.*, 1986).

In addition to motor neurons, other neurons make direct and indirect contact with skeletal muscle. For example, type Ia afferents, involved in proprioception, are located in the dorsal root ganglia (DRG) and form distal contacts with muscle spindles as well as proximal contacts with interneurons and motor neurons in the spinal cord (Kucera and Warlo, 1992; Snider *et al.*, 1992). Spinal motor neurons are a major target of spinal interneurons and the observation that interneurons are ablated in Isl-1-deficient mice lacking motor neurons has led to the suggestion that motor neuron generation is required for the subsequent differentiation of Engrailed-1 (En-1) interneurons (Pfaff *et al.*, 1996). In addition, the giant pyramidal cells of motor cortex, involved in fine motor control, project down the spinal cord and form contacts primarily with interneurons as well as with a small number of motor neurons. Proprioceptive neuron cells have also been suggested to require neurotrophic factors for their survival (Ernfors *et al.*, 1994; Klein *et al.*, 1994; Ringstedt *et al.*, 1997), whereas the trophic requirements of giant pyramidal cells remain undefined.

The myogenic regulatory factors (MRFs), a group of basic helix-loop-helix (bHLH) transcription factors consisting of MyoD, myogenin, Myf-5, and MRF4, play essential regulatory functions in the skeletal–muscle developmental program. The introduction of null mutations in *Myf-5*, *MyoD*, *myogenin*, and *MRF4* into the germline of mice has revealed the hierarchical relationships existing among the MRFs and established that functional redundancy is a feature of the MRF regulatory network (reviewed by Rudnicki and Jaenisch, 1995; Megeney and Rudnicki, 1995). Importantly, the entire embryonic lineage that gives rise to skeletal muscle never forms in compound-mutant animals lacking both *Myf-5* and *MyoD* (designated *Myf-5−/−:MyoD−/−*), as evidenced by the absence of myoblasts and myofibers throughout development. Consequently, *Myf-5−/−:MyoD−/−* pups are born, but are immobile and die soon after birth (Rudnicki *et al.*, 1993; B. Kablar and M. A. Rudnicki, unpublished).

In this study, we examined the development of motor system neurons in compound-mutant embryos lacking both *Myf-5* and *MyoD*. In the absence of skeletal myogenesis, we observed the normal birth of motor neurons in the spinal cord followed by their progressive loss. By contrast, the development of En-1- and Chx-10-expressing interneurons was unaffected. Motor neurons in the facial nucleus were almost entirely ablated. Severe deficits were also noted in the survival of proprioceptive sensory neurons in the dorsal root and, possibly, trigeminal ganglia. Pyramidal neurons in the E17.5 motor cortex appeared completely absent without any corresponding evidence of apoptosis. Taken together, these results indicate that the development of motor system neurons from the spinal cord to the motor cortex is tightly coupled to myogenesis.

MATERIALS AND METHODS

Interbreeding and Collection of Embryos

Embryos lacking both *Myf-5* and *MyoD* were derived by a two-generation breeding scheme. First, *MyoD−/−* mice were bred with *Myf-5+/−* mice to generate *Myf-5+/−:MyoD+/−* mice. Second, *Myf-5+/−:MyoD+/−* mice were interbred to obtain embryos of nine different genotypes as described in Rudnicki *et al.* (1993). Embryos and the fetal portion of the placenta were collected by cesarean section between embryonic day (E) 10.5 and E18.5 and embryos prepared for immunohistochemistry as described below. Genomic DNA was isolated from the fetal portion of the placenta using the procedure of Laird *et al.* (1991). Embryos were genotyped by Southern analysis (Sambrook *et al.*, 1989) of placental DNA using *Myf-5* and *MyoD* specific probes as described previously (Rudnicki *et al.*, 1993). Care of animals was in accordance with institutional guidelines.

Immunohistochemical and TUNEL Analysis

Myf-5−/−:MyoD−/− and wild-type embryos were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The head was removed from E14.5 and older embryos to allow proper fixation of the tissue. After fixation for 2–12 h (depending on the embryonic size) the embryos were either embedded in Histo Prep (OCT, Fisher Scientific) or dehydrated and embedded in paraffin wax. Transverse serial sections of the trunk and coronal or sagittal serial sections of the head were cut at 4 μm for immunohistochemical methods and at 7 μm for hematoxylin–eosin (HE) and cresyl fast violet (Nissle) staining (Lowe and Cox, 1990), using a rotary microtome or cryotome.

Immunohistochemical analysis was done as previously described (Rudnicki *et al.*, 1993) using mouse monoclonal anti-NF160 antibody (Sigma), mouse monoclonal anti-Islet-1/2 antibody (39.4D5, Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit polyclonal anti-Engrailed-1 antibody (anti-Enhb-1, kindly provided by A. L. Joyner), and rabbit polyclonal anti-Chx-10 antibody (kindly provided by T. M. Jessell). Primary antibodies were diluted as follows: 1:20, 1:5, 1:100, and 1: 100, respectively.

To detect apoptotic nuclei *in situ* by TUNEL (Gavrieli *et al.*, 1992), we employed the ApopTag detection system (Oncor and Genzyme), according to the manufacturer's instructions.

Morphometric Analysis

Morphometric analysis was performed on transverse sections through the level of upper thoracic (T1-4) and brachial (C4-7) spinal cord and on sagittal and horizontal sections through the head. In the younger embryos (E10.5–E14.5) motor neuron counts were made in every 10th section, while in older embryos (E15.5–E17.5) cell counts of motor neurons were made in every 20th section of just the spinal cord segments (with DRG) corresponding to the upper thoracic or brachial spinal cord level or the head sections corresponding to the FMN, trigeminal ganglion (TG), or the true motor cortex (the precentral region). The segment of the spinal cord was defined by a combination of anatomical criteria (e.g., counting of the somites) and dissected out before the embryo was embedded, whereas the FMN, TG, and the precentral region (the true motor cortex) were identified on the base of their anatomical localization in the serial sections of the embryonic head. Motor or sensory neurons of the head were identified by their large size of nucleus containing at least one nucleolus and abundant cytoplasm, while neurons in the spinal cord were identified by immunostaining to Isl-1/2. Isl-1/2-expressing motor neurons were counted under high magnification (400−600×) and, therefore, even nuclei that expressed lower levels of Isl-1/2 protein were noticed and counted. The correction factors for split nucleoli (Abercrombie, 1946; Clarke, 1993) were not employed and thus the data presented are the actual cell counts multiplied by either 10 or 20 and then divided by the total number of sections per region to yield cells per section. An analogous procedure was also performed on sections stained with cresyl fast violet (Nissle staining) on embryos between E15.5 and E17.5.

RESULTS

Development of Motor Neurons in the Spinal Cord Is Completely Dependent on Myogenesis

To determine whether the development of motor neurons in the spinal cord was perturbed in the absence of myogenesis, we performed immunohistochemistry with mouse monoclonal antibody 39.4D5 reactive with Islet-1 and Islet-2 (Isl-1/2) on wild-type and *Myf-5−/−:MyoD−/−* embryos. Isl-1 and Isl-2 are LIM-domain transcription factors and newly differentiated motor neurons first express Isl-1 followed by Isl-2 (Tsuchida *et al.*, 1994). Cells expressing Isl-1/2, and therefore committed to a motor neuron fate, are first situated in the single ventrolateral column (Ericson *et al.*, 1992; see Fig. 1A). Consequently, we could not discriminate between somatic (innervating skeletal muscle) and visceral motor neurons (innervating smooth muscle) at earlier embryonic ages (E10.5–E12.5). Morphometric analysis of sections prepared for immunohistochemistry against Isl-1/2 revealed that the position and appearance of motor neurons between E10.5 and E12.5 in *Myf-5−/−:MyoD−/−* embryos appeared indistinguishable from that of control wild-type littermates (Figs. 1A, 1B, and 4A and data not shown). Therefore, we conclude that the early inductive events on or before E12.5 that lead to the determination and differentiation of motor neurons occur independently of myogenesis.

A 60% reduction in the numbers of motor neurons in the brachial and lumbar ventral horn of the spinal cord between E13 and E18 has been suggested as typical during mouse embryogenesis (Lance-Jones, 1982; Oppenheim *et al.*, 1986; Oppenheim, 1991). Indeed, morphometric analysis of transverse sections of wild-type brachial spinal cord revealed that the total number of Isl-1/2-expressing motor neurons per section decreased by 51% between E12.5 and E14.5 with the reduction occurring primarily between E13.5 and E14.5 (Figs. 2 and 4E). The brachial segments of wild-type spinal cord contained 76 ± 4 Isl-1/2-positive cells per section at E12.5 (Fig. 4E), and this level decreased to 39 ± 6 Isl-1/2-positive cells per section by E14.5 (Fig. 4E). By contrast to wild-type embryos, the total number of Isl-1/2-expressing motor neurons in *Myf-5−/−:MyoD−/−* embryos decreased by 91% from 76 ± 5 per section at E12.5 (Fig. 4E), to 7 ± 5 per section at E14.5 (Fig. 4E), with the reduction occurring primarily between E12.5 and E13.5. However, morphometric analysis of transverse sections of wild-type thoracic spinal cord revealed that the total number of Isl-1/2-expressing motor neurons per section decreased by 41% between E12.5 and E17.5, with the reduction occurring at about a constant level (3–18%) between the embryonic ages (Figs. 1C, 1E, 1G, 4A, 4C, and 4D). The upper thoracic segments of wild-type spinal cord contained 91 ± 9 Isl-1/2-positive cells per section at E12.5 (Figs. 1A and 4A), and this level decreased to 54 ± 5 Isl-1/2-positive cells per section by E17.5 (Figs. 1G and 4A). By contrast to wild-type embryos, the total number of Isl-1/2-expressing motor neurons in *Myf-5−/−:MyoD−/−* embryos decreased by 78% from 95 ± 15 per section at E12.5 (Figs. 1B and 4A) to 21 ± 7 per section at E17.5 (Figs. 1H and 4A), with the maximum reduction occurring between E12.5 and E13.5 (69%), during the time that the three motor columns are elaborated.

At later stages (after E12.5) the visceral motor neurons, projecting to the sympathetic ganglia that control the activity of a variety of structures including smooth muscle, migrate away from the common motor column and form the so-called column of Terni (chick embryo) or visceral motor column (VMC) located in the centromedial region of the thoracic spinal cord gray matter (Ericson *et al.*, 1992). Somatic motor neurons at the thoracic level become localized into two ventrolateral columns called the medial and lateral median motor columns (MMC_m and MMC_l), innervating axial and body wall musculature, respectively (Tsuchida *et al.*, 1994). The lateral motor column (LMC) can also be distinguished at this embryonic age, but is located at the level of either brachial or lumbar enlargement of the spinal cord (Tsuchida *et al.*, 1994). The pattern of Isl-1/2 immunostaining obtained on sections from mouse embryos (Figs. 1 and 2) was very similar to that described for the chick embryos (Ericson *et al.*, 1992; Tsuchida *et al.*, 1994). Therefore, at embryonic stages E13.5 to E17.5 it was possible to distinguish four regions of Isl-1/2-expressing neurons consisting of the VMC (thoracic level only), containing visceral motor neurons, and the MMC_m, MMC_l (cervical and thoracic levels), and LMC (brachial and lum-

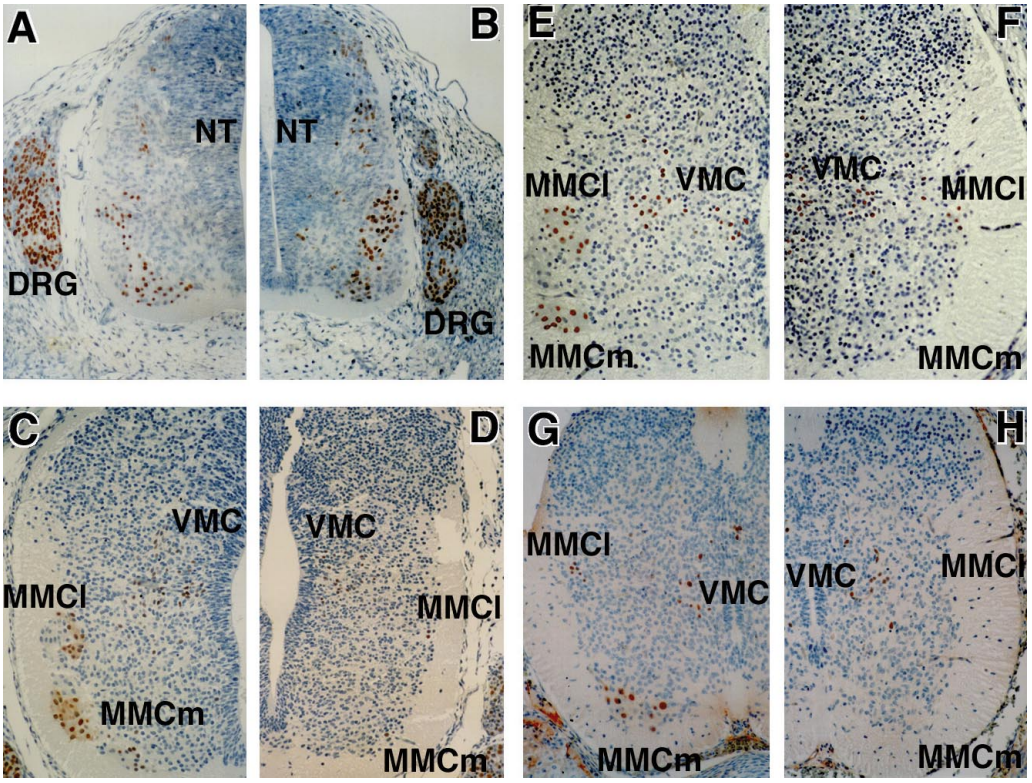


FIG. 1. Normal birth and subsequent ablation of Isl-1/2-expressing somatic motor neurons at the level of the upper thoracic spinal cord in the absence of myogenesis. Transverse sections of wild-type (A, C, E, G) and mutant (B, D, F, H) embryos at E12.5 (A, B), E13.5 (C, D), E15.5 (E, F), and E17.5 (G, H). Sections were immunostained with anti-Isl-1/2 antibody to reveal the distribution of Isl-1/2-expressing motor neurons in the neural tube (NT) and sensory neurons in the dorsal root ganglia (DRG) and to reveal somatic motor neurons in the MMC_m and MMC_l and visceral motor neurons in the VMC. The number of VMC neurons remained relatively constant in both wild-type and mutant embryos, whereas MMC neurons were absent by E16.5 (see Figs. 4C and 4D). Magnification, 200×.

bar levels), containing somatic motor neurons (Figs. 1C, 1E, 1G, 2A, 2C, and 2E).

At the level of the upper thoracic spinal cord the MMC_m of E13.5 wild-type embryos contained 39 ± 8 neurons per section and this number decreased to 21 ± 5 by E17.5 (Figs. 1C, 1E, 1G, and 4C). By contrast, the MMC_m of *Myf-5−/−:MyoD−/−* embryos only contained 3 ± 2 neurons per section at E13.5, and no Isl-1/2-expressing neurons were detectable in the MMC_m by E16.5 and E17.5 (Figs. 1D, 1F, 1H, and 4C). The MMC_l of E13.5 wild-type embryos contained 24 ± 6 neurons per section and this number decreased to 13 ± 4 by E17.5 (Figs. 1C, 1E, 1G, and 4D). By contrast, the MMC_l of E13.5 mutant embryos contained 3 ± 2 neurons per section and no Isl-1/2-expressing neurons were detectable by E16.5 and E17.5 (Figs. 1D, 1F, 1H, and 4D). Importantly, Nissle staining, immunohistochemistry with Isl-1/2, and NF160 antibody reactive with neurofilament revealed a complete absence of somatic motor neurons in the ventral and lateral horns of E18.5 *Myf-5−/−:MyoD−/−* embryos (not shown). The numbers of visceral motor neurons (between 20 ± 6 and 25 ± 4) in the wild-type and mutant VMC were similar and did not significantly change between E13.5 and E17.5 (Figs. 1C–1H, numerical data not shown). Similar analyses were also performed on Nissle-stained sections from E15.5 to E17.5 embryos and results obtained were analogous to that observed with Isl-1/2 immunostaining (not shown).

During the development of the nervous system, it is believed that motor neurons that fail to form functional contacts with muscle undergo normal programmed cell death or apoptosis due to an absence of trophic support from muscle (Lance-Jones, 1982; Oppenheim *et al.*, 1991, 1992, 1995; Sendtner *et al.*, 1992a,b; Yan *et al.*, 1992, 1993, 1994, 1995; Henderson *et al.*, 1993, 1994; Koliatsos *et al.*, 1993; Oppenheim, 1996). Therefore, to determine whether the loss of motor neurons in *Myf-5−/−:MyoD−/−* embryos was due to apoptosis, we performed TUNEL (terminal transferase-mediated dUTP-biotin nick end labeling; Gavrieli *et al.*, 1992) staining on sections of spinal cord at different developmental stages (Fig. 3).

In wild-type embryos between E10.5 and E17.5 we observed between one and four apoptotic bodies per section in

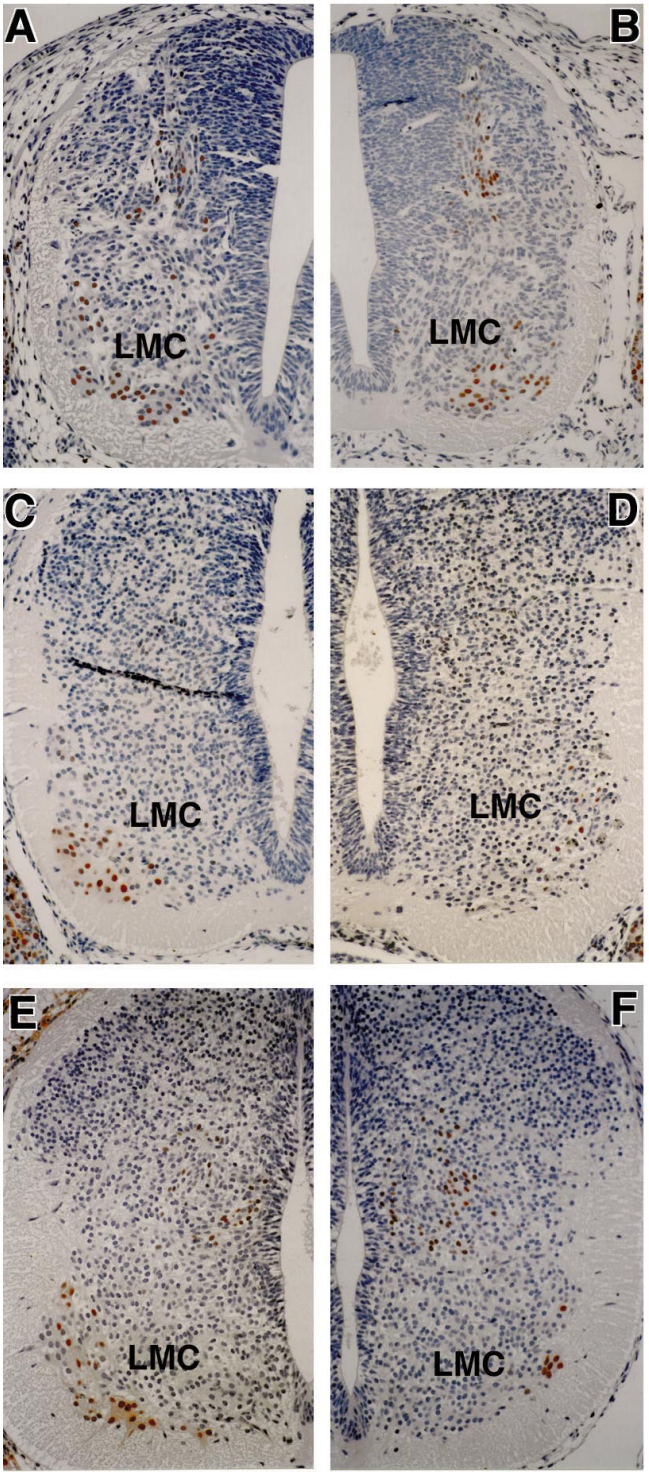


FIG. 2. Normal birth and subsequent ablation of Isl-1/2-expressing somatic motor neurons at the level of brachial spinal cord in the absence of myogenesis. Transverse sections of wild-type (A, C, E) and mutant (B, D, F) embryos at E12.5 (A, B), E13.5 (C, D), and E14.5 (E, F). Sections were immunostained with anti-Isl-1/2 antibody to reveal the distribution of Isl-1/2-expressing somatic motor neurons in the LMC. The number of LMC neurons was normal at E12.5 (see Fig. 4E) in both wild-type and mutant embryos. By E14.5 the number of LMC neurons decreased by 51% (see Fig. 4E) in wild-type embryos and by 91% in mutant embryos (see Fig. 4E). Magnification, 200×.

the anterior half of the brachial and thoracic spinal cord (Figs. 3A, 3C, 3E, 4B, and 4F), confirming that during normal development some cells in the spinal cord undergo programmed cell death. In *Myf-5−/−:MyoD−/−* embryos between E10.5 and E12.5, we observed similar low numbers of apoptotic bodies in the spinal cord (Figs. 4B and 4F). However, mutant embryos exhibited a significant increase in the number of apoptotic bodies with 22 ± 1 observed per section on E13.5 in the region of LMC (brachial spinal cord, Figs. 3B and 4F), 18 ± 2 observed per section on E13.5 in the region of MMC_m and MMC_l (thoracic spinal cord, Figs. 3D and 4B), and 13 ± 2 and 10 ± 2 observed per section on E15.5 (Figs. 3F and 4B) and E16.5 (Fig. 4B), respectively, mostly in the region of MMC_m (thoracic spinal cord). Therefore, the number of apoptotic bodies in the mutant spinal cord at E13.5, E15.5, and E16.5 correlated with the decrease in numbers of Isl-1/2-expressing motor neurons in the mutant LMC, MMC_m, and MMC_l.

A subset of interneurons express either the homeodomain protein En-1, so-called V1 class of interneurons, or the homeodomain protein Chx-10, so-called V2 class of interneuros (Ericson *et al.*, 1997). Later in development interneurons are located in the ventrolateral spinal cord intermingled with Isl-1/2-expressing somatic motor neurons (Pfaff *et al.*, 1996; Matisse and Joyner, 1997; Ericson *et al.*, 1997). The development of interneurons has been suggested to be dependent on the presence of Isl-1-expressing motor neurons as interneurons are ablated in mice lacking Isl-1 in which motor neurons fail to differentiate (Pfaff *et al.*, 1996). Immunohistochemical analysis of E17.5 embryos with anti-Enhb-1 reactive against En-1 and anti-Chx-10 revealed that neither V1 nor V2 interneurons were affected by the massive programmed cell death that resulted in a complete ablation of Isl-1/2-expressing somatic motor neurons at the upper thoracic (Fig. 5) and brachial (not shown) levels of the mutant spinal cord. Per section, we found 8 ± 2 En-1-positive nuclei in the wild-type and 12 ± 4 in the mutant spinal cord and 14 ± 2 Chx-10-positive nuclei in the wild-type and 17 ± 4 in the mutant spinal cord. Taken together, these data suggest that motor neurons are not required for the survival of differentiated interneurons, during later stages of embryonic development.

Deficiency in Neurite Outgrowth in Myf-5−/−: MyoD−/− Embryonic Development

Segmentation of axon outgrowth from the spinal cord was examined on serially sectioned *Myf-5−/−:MyoD−/−* and wild-type E11.5 and E12.5 embryos, immunostained

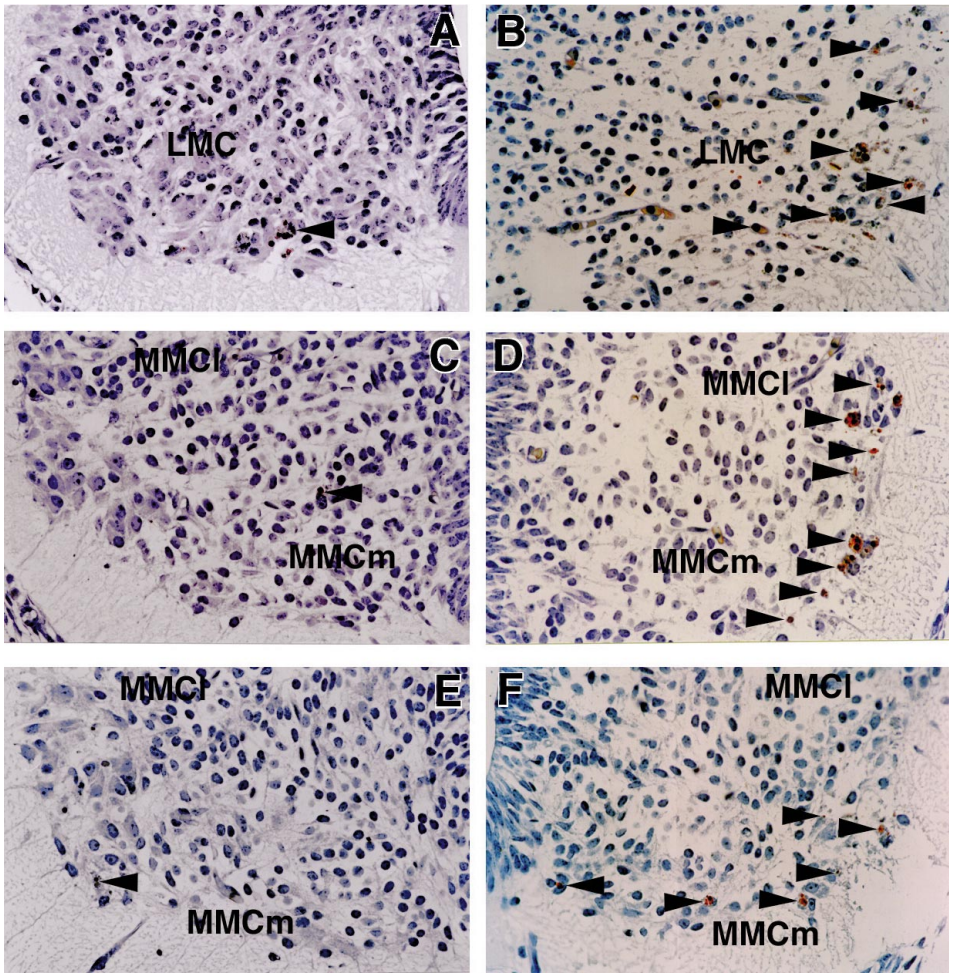


FIG. 3. Elevated apoptosis in the spinal cords of E15.5 *Myf-5−/−:MyoD−/−* embryos. TUNEL staining was performed on transverse sections to detect apoptotic bodies from wild-type (A, C, E) and mutant (B, D, F) embryos at E13.5 [(A, B) at the brachial level and (C, D) at the thoracic level] and E15.5 [(E, F) at the thoracic level]. A low level of apoptosis was observed in the ventral horn of the spinal cord of wild-type embryos, whereas massive apoptosis was observed in *Myf-5−/−:MyoD−/−* embryos (see Figs. 4B and 4F). Apoptotic bodies are indicated with arrowheads. Magnification, 400×.

with antibody against neuron-specific 160-kDa neurofilament protein (NF160). The patterns of spinal nerves and spinal nerve plexuses at the forelimb level were found to be in their normal position and relationships in the mutant embryos (Figs. 6A and 6B and data not shown). By contrast, the development of the dorsal ramus, a branch of the spinal nerve that diverges dorsally to innervate epaxial muscle and dermis, was severely reduced (Figs. 6C–6F). In serial sections of wild-type E12.5 embryos, the course of the epaxial and cutaneous components of the dorsal ramus was readily followed (Figs. 6A, 6C, and 6E). By contrast, in the mutant E12.5 embryos (Figs. 6B, 6D, and 6F), only a stump of the dorsal ramus was evident on the spinal nerve (Figs. 6B and 6D, arrowhead). Therefore, motor neurons that normally innervate epaxial muscle did not project their axons to the field normally occupied by their targets. Moreover, the cutaneous nerves that project to the dorsal dermis also failed to reach their targets (Fig. 6F).

In serially sectioned mutant E12.5 embryos, the axons of LMC neurons were observed to project through the trunk toward the forelimbs in a normal manner. Moreover, the ventral and dorsal characters of the main forelimb nerve branches were readily discerned (Figs. 6G and 6H). However, the finer branches that normally provide innervation to the forelimb musculature were notably absent (Figs. 6I and 6J), while cutaneous branches normally developed (Figs. 6K and 6L).

Our data suggest that the proximity of the myotome provides a cue for the outgrowth of projections from both motor and sensory neurons in the dorsal ramus. On the contrary, the presence of the limb muscle anlagen appears to provide a cue for the outgrowth of secondary branches of

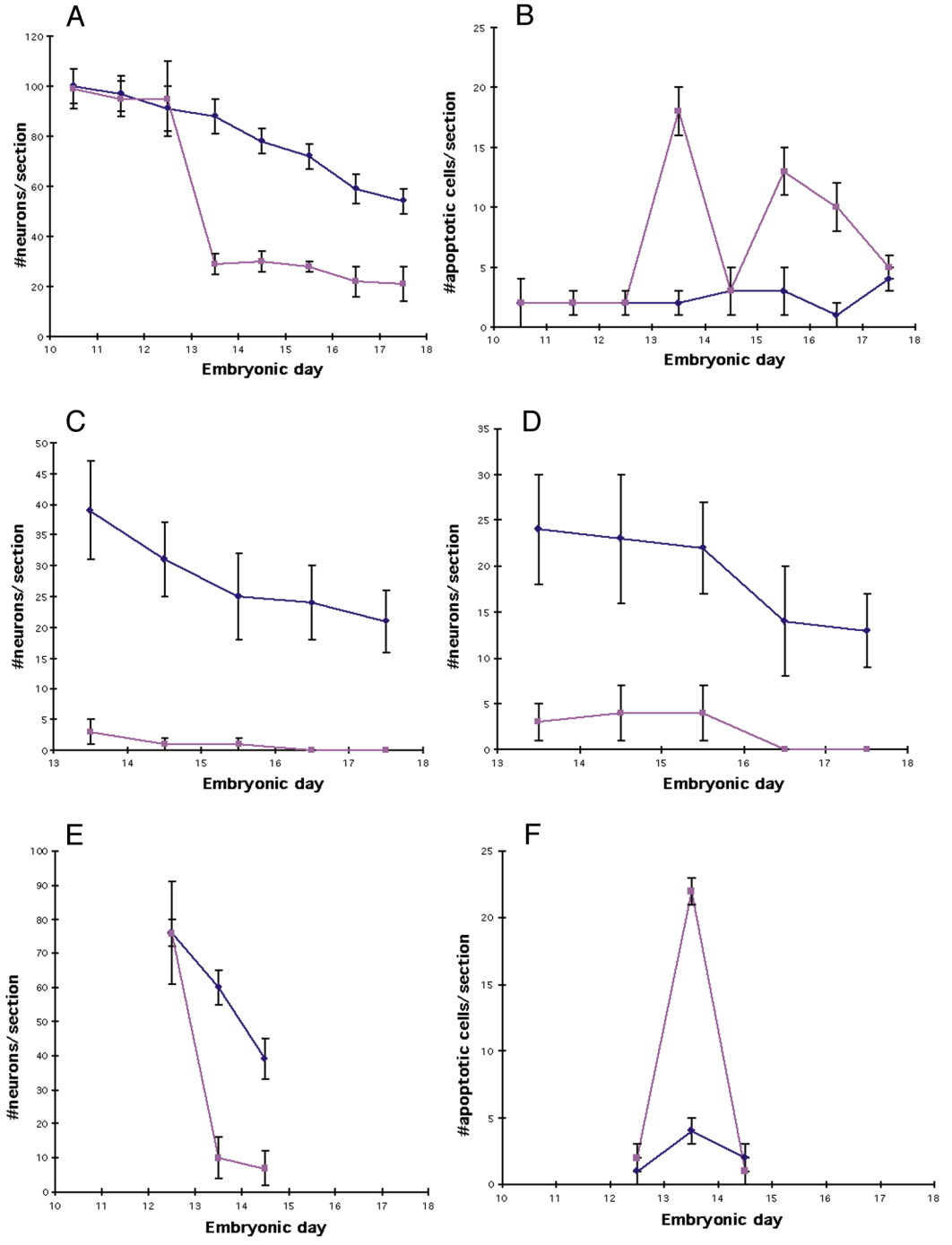


FIG. 4. Morphometric analysis of neurogenesis in the absence of skeletal muscle. (A) Total number of Isl-1/2-expressing motor neurons in the thoracic spinal cord. (B) Number of apoptotic cells in the regions of the MMCm and the MMCl of the thoracic spinal cord. (C) Number of Isl-1/2-expressing motor neurons in the MMCm of the thoracic spinal cord. (D) Number of Isl-1/2-expressing motor neurons in the MMCl of the thoracic spinal cord. (E) Number of Isl-1/2-expressing motor neurons in the LMC of the brachial spinal cord. (F) Number of apoptotic cells in the region of the LMC of the brachial spinal cord. Frequency of Isl-1/2-expressing motor neurons and apoptotic bodies are expressed as the mean and standard error of the mean. Wildtype frequencies are plotted in dark purple and mutant frequencies are plotted in light purple. Statistically significant differences were detected between E13.5 and E17.5 in A, on E13.5, E15.5 and E16.5 in B, between E13.5 and E17.5 in C and D, on E13.5 and E14.5 in E and on E13.5 in F [t test ($P < 0.001$)].

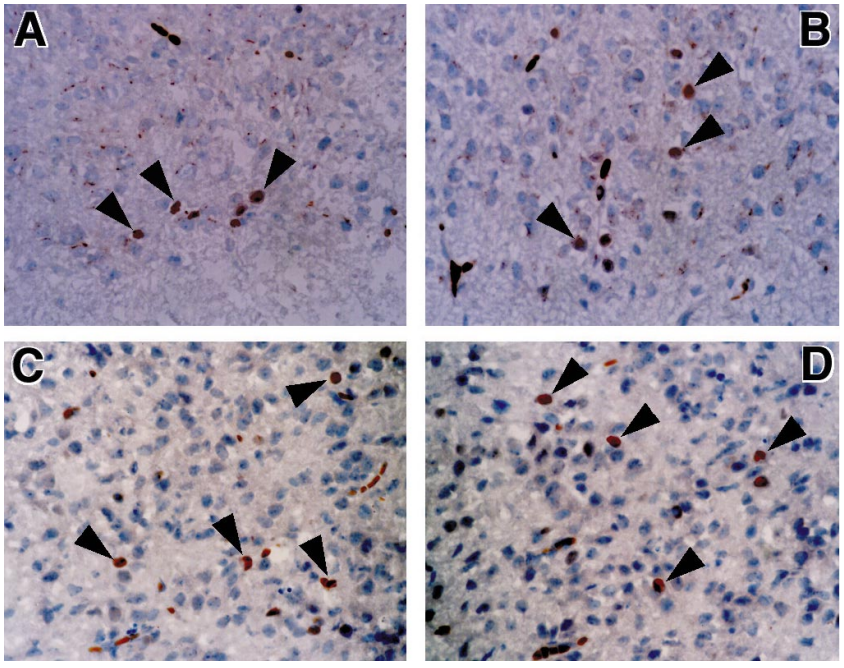


FIG. 5. Unaffected En-1 and Chx-10 interneuros. Transverse frozen sections of wild-type (A, C) and mutant (B, D) embryos at E17.5. Sections were immunostained with anti-En-1 (A, B) and anti-Chx-10 (C, D) antibodies to reveal a subset of interneurons in the ventrolateral spinal cord. Neither group of interneurons was affected in the absence of somatic motor neurons at the level of upper thoracic spinal cord. En-1 and Chx-10 interneurons are indicated with arrowheads. Magnification, 400×.

the motor nerves from the dorsal and ventral characters of the main forelimb nerve, but cutaneous sensory branches develop independently on myogenesis. Taken together, these data indicate that while the formation of the main branches of nerves occurs independently of myogenesis, the outward growth of the finer projections of both motor and sensory nerves appears entirely dependent on myogenic development at proximal sites, but not at distal sites.

Ablation of Proprioceptive Sensory Neurons in the Absence of Myogenesis

To determine whether the development of neural structures of neural crest origin was affected in the absence of the myotome, we examined serial sections stained with antibody NF160 reactive against neurofilament, as well as Nissle-stained sections. The segmentation and size of the sympathetic ganglia and preaortic ganglia as well as the development of the suprarenal gland and organ plexuses (e.g., in the gut) were not altered in the absence of the myotomal muscle differentiation between E11.5 and E12.5 and on E17.5 (not shown). Therefore, we conclude that the migration and fate of neural crest cells that form the segmentally arranged ganglia of the sympathetic nervous system were not altered by an absence of myogenic development.

During development, innervation of myofibers by proprioceptive neurons in the DRG induces the formation of muscle spindles (Kucera and Warlo, 1992). Subsequently, the dorsal root afferents and motor neuron dendrites begin to form contacts in the spinal cord around day 17 of gestation (Snider *et al.*, 1992). Most DRG neurons are small-diameter cutaneous afferents for nocioception and thermoception, while the comparatively small population (~14–19%) of large-diameter DRG neurons supply muscle spindles and are primarily involved in proprioception (Oakley *et al.*, 1997; Wright *et al.*, 1997). To investigate the consequence of the absence of somatic motor neurons and muscle spindles on development of the proprioceptive sense organ of the mutant embryos, we performed morphometric analysis on Nissle- and NF160-stained sections of DRG at the level of the upper thoracic spinal cord, from embryos of different developmental stages.

Morphometric analysis of brachial DRG of embryos between E11.5 and E15.5 revealed that the segmental arrangement and overall size of the DRG of *Myf-5−/−:MyoD−/−* embryos were indistinguishable from wild-type embryos (Figs. 1A and 1B and data not shown). For example, the DRG of wild-type embryos contained 120 ± 2 neurons per section at E10.5 and this increased to 153 ± 6 by E15.5, whereas mutant DRG contained 118 ± 2 neurons at E10.5 and this increased to 150 ± 14 by E15.5. By E16.5 we observed a somewhat decreased number of DRG neurons in both wild-type and mutant embryos. Therefore, the migration

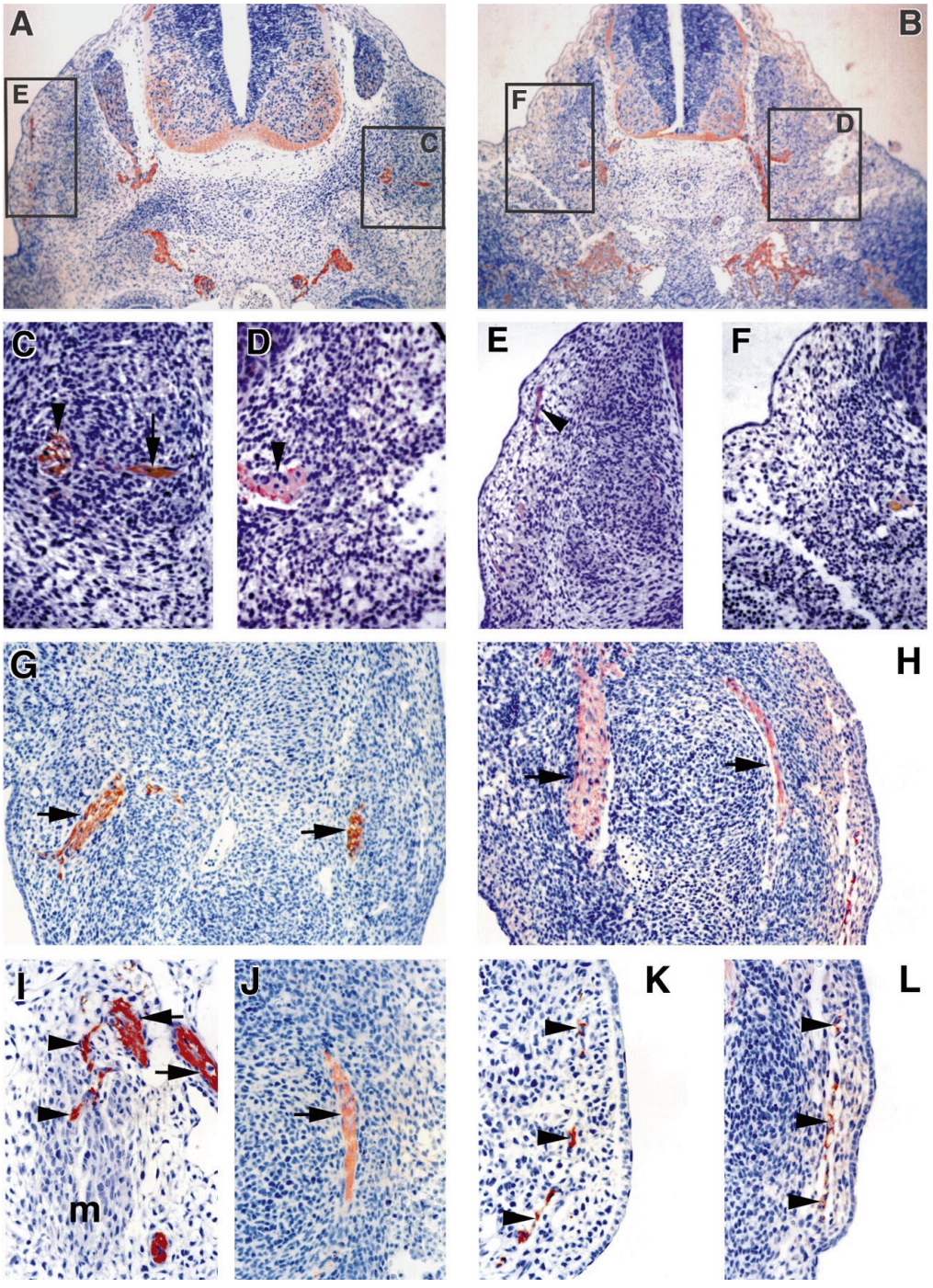


FIG. 6. Abnormal outgrowth of nerves in *Myf-5−/−:MyoD−/−* embryos. The routes of innervation were followed in NF160-immunostained serial sections of wild-type (A, C, E, G, I, K) and mutant (B, D, F, H, J, L) E12.5 embryos. Brachial spinal nerves and plexuses are shown in wild-type (A) and mutant (B) embryos, and the insets (C–F) show detail. The dorsal ramus (arrowhead and arrow in C) was reduced to a stump in mutant embryos (arrowhead in D). The epaxial muscle component of the dorsal ramus (arrow in C) was absent in mutant embryos (D), and the cutaneous component of the dorsal ramus (arrowhead in E) was absent in mutant embryos (F). The ventral and dorsal characters of the main forelimb nerve branches (arrows) were readily discerned in both wild-type (G) and mutant (H) embryos, but the finer branches (arrowheads in I) that normally provide innervation to the forelimb musculature (m) were notably absent in mutant embryos (compare I and J). Cutaneous branches normally developed in mutant forelimbs (arrowheads in K and L). Magnification, 100× (A, B); 200× (G, H); and 400× (C–F and I–L).

and differentiation of neural crest cells that form the DRG occur independently of myogenesis. However, by E17.5 we observed markedly decreased numbers of large, mostly proprioceptive, neurons in the DRG of *Myf-5−/−:MyoD−/−* embryos, with 25 ± 3 per section in wild-type embryos and 2 ± 1 per section in mutant embryos (Figs. 7A and 7B). By contrast, the number of small DRG neurons, which mediate nocioceptive and other sensory modalities, was similar between wild-type and mutant DRG (Figs. 7A and 7B).

TUNEL analysis of serial sections through DRG of wild-type and *Myf-5−/−:MyoD−/−* embryos revealed similar levels of apoptosis from E10.5 to E16.5. However, by E17.5 we observed markedly increased numbers of apoptotic bodies in the DRG of *Myf-5−/−:MyoD−/−* embryos, with 2 ± 1 per section in wild-type DRG and 10 ± 1 per section in mutant DRG (Figs. 7C and 7D). The late loss of proprioceptive neurons in the DRG of *Myf-5−/−:MyoD−/−* embryos suggests that cell death occurs just after the time that proprioceptive neurons would normally form contacts with motor neurons in the spinal cord (Ernfors and Persson, 1991).

Another important sensory organ is the TG that lies in a dural pouch called the cavum trigeminale (Figs. 7G and 7H). The TG gives off three nerves, the ophthalmic, the maxillary, and the mandibulary, innervating the ocular muscles and jaw muscle, respectively. The TG does not contain proprioceptive sensory neurons because fibers of the mesencephalic tract that carry proprioceptive impulses from the muscles of mastication arise from the mesencephalic nucleus in the brain stem. However, the primary proprioceptive neurons of extraocular muscles are believed to be situated in the TG (Daunicht *et al.*, 1985; Pettorossi *et al.*, 1995). Retrograde horseradish peroxidase studies on afferent innervation of extraocular muscles in the rat have revealed that about 18–38 proprioceptive neurons per section may be present in the TG (Daunicht *et al.*, 1985). Thus, if the TG contains proprioceptive neurons we would expect to observe some neuronal loss analogous to that observed in DRG.

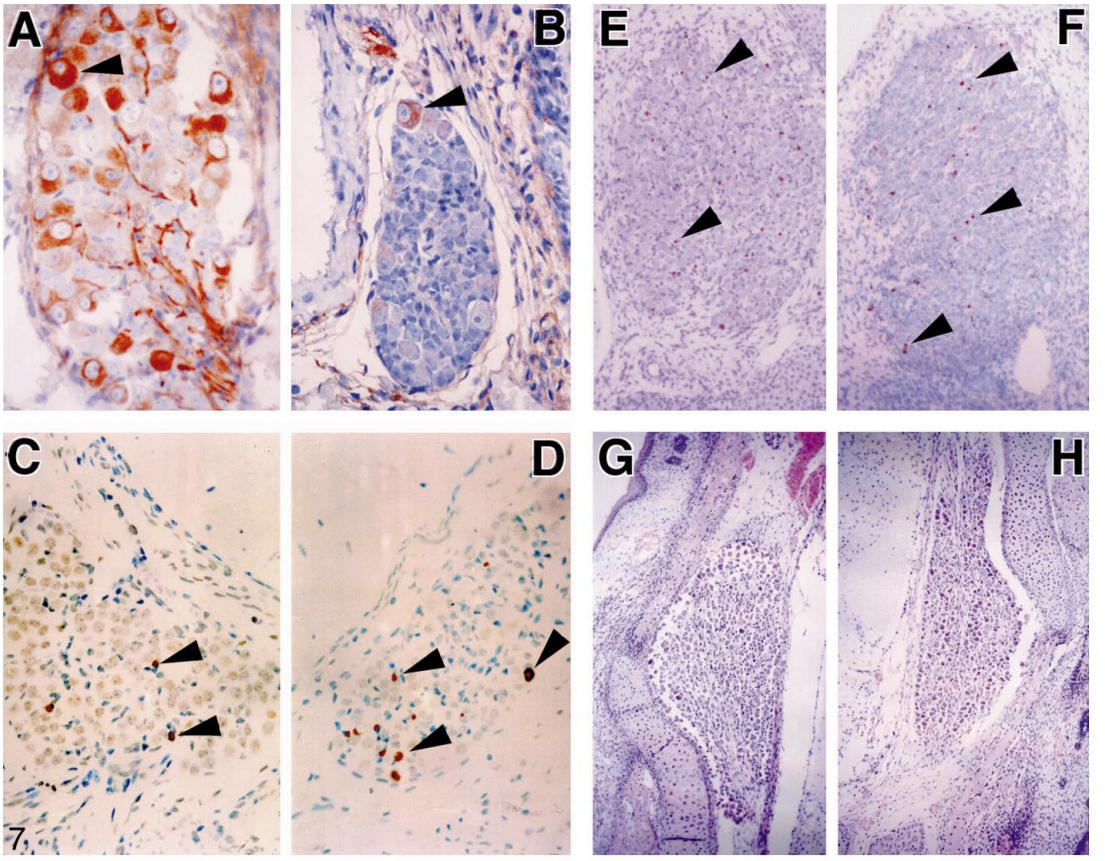
Detailed analysis of HE-stained serial sagittal sections through the head of the mutant embryos revealed that the appearance of the TG was not significantly affected in the mutant embryos (Figs. 7G and 7H). Morphometric analysis on mutant E17.5 embryos revealed only a small 4% decrease in the number of neurons in the TG. Representative sections were examined by TUNEL analysis. While abundant numbers of apoptotic bodies were observed in both wild-type and mutant TG at E15.5 (Figs. 7E and 7F), almost no apoptosis was observed in either wild-type or mutant TG by E17.5 (not shown). Therefore, unlike the DRG we did not observe a significant loss of sensory neurons in the TG in the absence of myogenesis, but that can be due to the low numbers of proprioceptive sensory neurons in the TG.

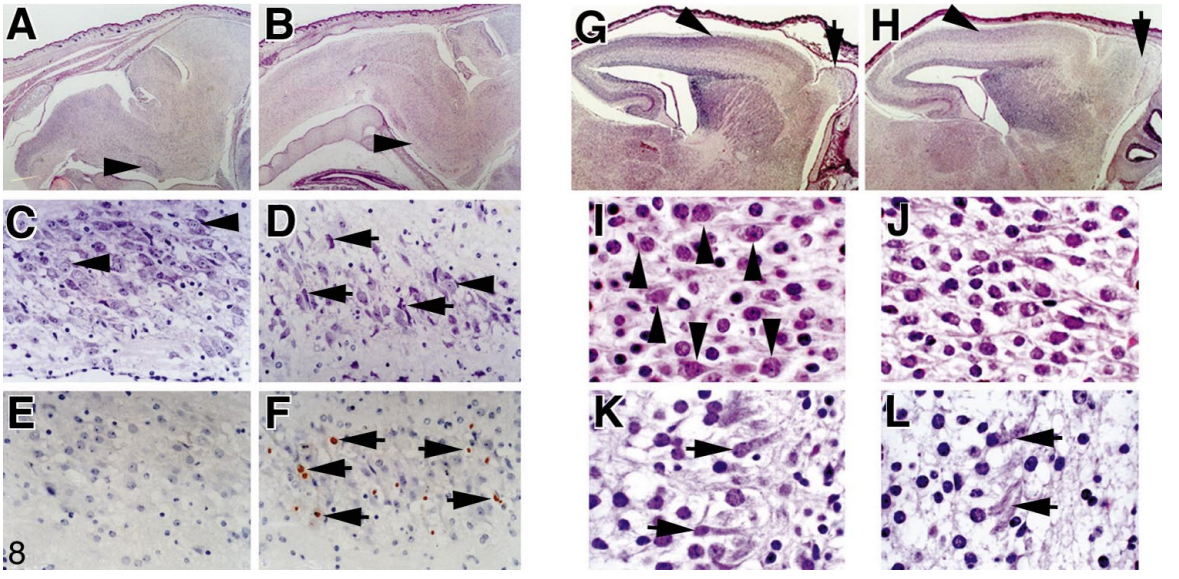
Massive Apoptosis in the Facial Motor Nucleus and a Complete Lack of Pyramidal Cells in the Motor Cortex in Term Myf-5−/−:MyoD−/− Embryos

The motor fibers to the mimetic muscles of the face stem from the large multipolar nerve cells of the facial motor nucleus (FMN). At E17.5, the FMN appears as a pear-shaped gray mass in the ventrolateral margin of the pons, located approximately half way between the medulla oblongata/pons border (pontine flexure) and the inferior olivary nucleus at the one side and pontine nuclei at the other side (Jacquin *et al.*, 1996). The FMN of wild-type animals contained ample numbers of the easily recognizable large multipolar neurons that innervate facial musculature (Figs. 8A and 8C). By contrast, detailed analysis of HE-stained serial sagittal sections through the mutant FMN revealed almost a complete absence of large multipolar neurons (Figs. 8B and 8D). Many of the remaining neurons exhibited decreased nuclear size, decreased numbers of nucleoli, and altered general appearance and staining characteristics.

FIG. 7. Type Ia sensory neurons undergo apoptosis in the absence of myogenesis. NF160-immunostained transverse serial sections of E17.5 embryos revealed a 12.5-fold decrease in large proprioceptive neurons (arrowheads) in the mutant DRG (B) in comparison to the wild-type DRG (A). Detection of apoptotic bodies by TUNEL revealed a significant 5-fold increase in the number of stained cells (arrowheads) in mutant DRG (D) relative to wild-type DRG (C). By contrast, TUNEL analysis of the trigeminal ganglia (TG) revealed no significant difference in the number of apoptotic cells (arrowheads) between wild-type (E) and mutant (F) E15.5 embryos. No TUNEL-positive cells were detected in the E17.5 TG and morphometry revealed no significant difference in the numbers of sensory neurons between wild-type (G) and mutant (H). Magnification, 400× (A–D); 150× (E, F); and 100× (G, H).

FIG. 8. Apoptotic loss of neurons in the facial motor nucleus and apparent absence of giant pyramidal cells in *Myf-5−/−:MyoD−/−* motor cortex. Sagittal sections through the brain of E17.5 wild-type (A, G) and mutant (B, H) embryos at the level of facial motor nucleus (FMN, C–F), the motor cortex (arrowheads in G and H), and the olfactory lobe (arrows in G and H).The insets detail the normal appearance of neurons in the FMN in wild-type brain (arrowheads in C) and the presence of numerous degenerating and pyknotic nuclei (arrows in D), and abnormal cells (arrowhead in D), in the FMN of mutant brain. TUNEL analysis of wild-type FMN (E) did not detect any apoptotic nucleus, while the mutant FMN (F) contained 20 ± 5 apoptotic bodies per section. The motor cortex of wild-type (I) embryos contained numerous giant pyramidal cells (arrowheads in I), whereas the motor cortex of mutant embryos clearly lacked any giant pyramidal cells (J). In contrast, sensory neurons (arrows) in the olfactory lobe appeared normal in both wild-type (K) and mutant (L) embryos. Magnification, 25× (A, B, G, H); 400× (C–F); and 1000× (I–L).





Moreover, the mutant FMN contained markedly increased numbers of degenerating and pyknotic neurons (Fig. 8D). TUNEL analysis of wild-type FMN failed to detect any apoptotic bodies (Fig. 8E), whereas the mutant FMN contained 20 ± 5 apoptotic bodies per section (Fig. 8F).

Sagittal serial sections through the brain of E15.5, E16.5, and E17.5 wild-type and *Myf-5−/−:MyoD−/−* embryos were analyzed by immunohistochemistry with NF160 antibody, TUNEL, or following HE or cresyl fast violet staining. The true motor cortex (the precentral region) was identified as a region of the frontal lobe situated in front of the central sulcus. At E17.5 the motor cortex was characterized by reduction or loss of the granular layers, an increase in the pyramidal layers, the exceptional thickness, and the presence of easily identifiable giant pyramidal neurons (Tsien *et al.*, 1996).

The motor cortex of E17.5 wild-type fetuses contained abundant numbers of pyramidal neurons (Figs. 8G and 8I). In contrast, the motor cortex of E17.5 *Myf-5−/−:MyoD−/−* animals clearly lacked any giant pyramidal cell recognizable by morphology alone (Figs. 8H and 8J). However, the number and distribution of olfactory neurons appeared normal (Figs. 8K and 8L). In wild-type and mutant E15.5 and E16.5 embryos it was not possible to identify giant pyramidal neurons on the base of their morphology alone. Importantly, the motor cortex of both wild-type and mutant brains contained virtually no apoptotic bodies at E15.5, E16.5, or E17.5 (not shown). Therefore, because we were unable to detect neither pyramidal neurons nor elevated programmed cell death in the motor cortex of mutant embryos, we suggest that the normal developmental program of pyramidal neurons requires myogenesis.

DISCUSSION

In mutant animals lacking both *Myf-5* and *MyoD*, the entire embryonic lineage that gives rise to skeletal muscle never arises (Rudnicki *et al.*, 1993; Kablar and Rudnicki, unpublished). In the absence of skeletal myogenesis, we observed the normal birth of Isl-1/2-expressing motor neurons in the spinal cord, followed by their progressive loss leading to complete ablation by birth. The loss of LMC, MMC_m, and MMC_l motor neurons was correlated with the presence of elevated apoptosis. Unexpectedly, the number of En-1- and Chx-10-expressing interneurons was not affected in mutant mice. In the DRG, the proprioceptive sensory neurons underwent apoptosis late in development as did the motor neurons in the facial motor nucleus. By contrast, giant pyramidal neurons in the motor cortex appeared to fail to differentiate. Taken together, these results indicate that motor system neurogenesis is tightly coupled to myogenesis through development.

Somatic motor neurons in the spinal cord of the mouse embryo have been suggested to undergo a large reduction in number between midgestation and birth (Lance-Jones, 1982; Oppenheim, 1986). Indeed, morphometric analysis of transverse sections of wild-type upper thoracic spinal cord revealed that the total number of Isl-1/2-expressing motor neurons per section decreased by 41% between E12.5 and E17.5. This reduction was noticed from E13.5 onward, when three motor columns became elaborated at the thoracic level of the spinal cord: the VMC was formed in the centromedial region of the thoracic spinal cord gray matter by motor neurons that migrated away from the common motor column (Ericson *et al.*, 1992), while somatic motor neurons became localized into two ventrolateral columns (MMC_m and MMC_l, Tsuchida *et al.*, 1994). Morphometric analysis in this study included all Isl-1/2-positive cells in sections of the upper thoracic spinal cord and, therefore, both somatic and visceral motor neurons were counted between E12.5 and E17.5. If only somatic motor neurons in the ventral horns were considered, without visceral motor neurons (Isl-1/2 stained cells were situated in the central–medial position of the spinal cord), the observed decrease in the motor neuronal number would be 63% (from 91 ± 9 at E12.5 to 34 ± 5 at E17.5) and, therefore, very close to the results reported by Lance-Jones (1982) and Oppenheim *et al.* (1986). However, morphometric analysis of transverse sections of wild-type brachial spinal cord revealed that already between E12.5 and E14.5 the total number of Isl-1/2-expressing motor neurons per section decreased by 51%. At the brachial level of the spinal cord only somatic motor neurons in the LMC were considered (visceral motor neurons are not present at this level of the spinal cord; Tsuchida *et al.*, 1994). Therefore, our results suggest that, at the level of the thoracic spinal cord, only about 41% of motor neurons were eliminated by cell death, while about 22% possibly just migrated away from the ventral horns to the central–medial part of the spinal cord to form the VMC.

Using a binary transgenic system based on Cre-mediated DNA recombination, Grieshammer *et al.* (1998) have been able to obtain a gradual ablation of skeletal muscle during mouse embryogenesis, beginning approximately on E12.5. By E18.5, embryos obtained using this method still contain some skeletal muscle (Grieshammer *et al.*, 1998). Analysis of hematoxylin–eosin-stained sections reveals that the gradual absence of skeletal muscle results in an incomplete loss of spinal motor neurons (Grieshammer *et al.*, 1998). By contrast, in the complete absence of skeletal muscle, the total number of Isl-1/2-expressing motor neurons in *Myf-5−/−:MyoD−/−* embryos decreased by 78% at the level of the thoracic spinal cord and by 91% at the level of the brachial spinal cord, and by birth, no somatic and only visceral motor neurons were detected in the spinal cord of mutant embryos. The numbers of visceral motor neurons in the wild-type and mutant VMC were similar and did not significantly change between E13.5 and E17.5. Therefore, we interpret our data to suggest that in the spinal cord of the mouse, the number of surviving motor neurons is tightly correlated with the number required to innervate the available muscle targets. Moreover, because normal numbers of Isl-1/2-expressing cells were detected in the *Myf-5−/−:MyoD−/−* spinal cord before E12.5, we conclude

that the early determination and differentiation of motor neurons occur independently of myogenesis.

It has been documented that the mechanism of selection of motor neurons is achieved by programmed cell death (Oppenheim *et al.,* 1991, 1992, 1995; Sendtner *et al.,* 1992a,b; Yan *et al.,* 1992, 1994, 1995; Henderson *et al.,* 1993, 1994; Koliatsos *et al.,* 1993; Oppenheim, 1996; Grieshammer *et al.,* 1998). In wild-type embryos between E10.5 and E17.5 we observed between one and four apoptotic bodies per section in the anterior half of the brachial and thoracic spinal cord, suggesting that during normal development cells in the spinal cord do undergo programmed cell death. In *Myf-5−/−:MyoD−/−* embryos between E10.5 and E12.5, we observed low numbers of apoptotic bodies in the spinal cord. However, mutant embryos exhibited a massive apoptosis on E13.5 at the level of brachial spinal cord in the region of LMC and at the level of thoracic spinal cord in the region of MMC_m and MMC_l. Slightly later, on E15.5 and E16.5, a significant increase in the number of apoptotic bodies was also observed at the level of thoracic spinal cord in the region of MMC_m. At that time (on and after E16.5) Isl-1/2-expressing somatic motor neurons were already completely absent in the spinal cord of mutant embryos. Therefore, the appearance of apoptotic bodies in the mutant spinal cord is correlated with the decrease in numbers of Isl-1/2-expressing motor neurons in the mutant LMC, MMC_m, and MMC_l, clearly providing evidence that programmed cell death is an underlying mechanism for matching the size of the innervating pool to the size of its target.

Moreover, formation of primary myofibers is completed in the mouse before E14 followed by an additional round of myogenesis forming the so-called secondary myofibers (Hauschka, 1994). The final number of motor neurons is believed to have a 1:1 relationship with the number of primary myofibers present during early development rather than being correlated with the number of myofibers in the adult muscle (Oppenheim, 1991; Brennan, 1996). By E14.5, we observed that the brachial spinal cord was almost completely devoid of somatic motor neurons in *Myf-5−/−:MyoD−/−* embryos. Therefore, our data are consistent with the notion that the early formation of primary myofibers is important in determining the final number of motor neurons.

Major synaptic targets of interneurons are the spinal motor neurons, together with other interneurons in the spinal cord and brain. In the rat, spinal interneurons are believed to undergo a brief period of programmed cell death beginning shortly after birth (Lawson *et al.,* 1997). However, in chicken embryos no evidence for a developmental reduction in numbers of interneurons is observed, and removal of targets does not induce apoptotic loss of interneurons (McKay and Oppenheim, 1991). In the absence of myogenesis and subsequent loss of motor neurons during the development of *Myf-5−/−:MyoD−/−* embryos, we observed that neither En-1- nor Chx-10-expressing interneurons were affected at the thoracic and brachial levels of the spinal cord. By contrast, mouse embryos lacking Isl-1 that fail to develop motor neurons also fail to develop En-1-expressing interneurons (Pfaff *et al.,* 1996). One interpretation of these results is that interneuronal development is positively regulated by Isl-1-expressing motor neurons at early developmental times. During late developmental ages the number of interneurons appears not to be dependent on the number of their motor neuronal targets (Fig. 5; Grieshammer *et al.,* 1998), including VMC neurons (interneurons are normal in mutant embryos even at the brachial level which normally does not contain VMC neurons). Therefore, the underlying mechanism that regulates interneuronal survival seems to be different from the target-dependent mechanisms that appear to regulate motor neuronal survival. Alternatively, interneurons may survive longer without their motor neuronal targets and undergo postnatal programmed cell death.

In embryos deficient for both *Myf-5* and *MyoD,* the dorsal ramus was reduced to a stump on the spinal nerve. While chick surgery experiments demonstrated that outgrowing neurites of the dorsal ramus respond specifically to the dermamyotome, it was not possible to conclusively determine whether growth cones were responding to cues from the myotome or the dermatome (Tosney, 1987). Our results strongly support the hypothesis that axons of motor and sensory neurons that form the dorsal ramus respond to cues provided by the myotome rather than the dermatome. The lack of both motor and sensory components of the dorsal ramus in the absence of myotome confirms that sensory axons require motor neurites for outgrowth (Tosney, 1987). Moreover, our data clearly demonstrate that the outgrowth, organization, and distal progression of the spinal nerves were unaffected during the development of *Myf-5−/−:MyoD−/−* embryos.

The development of the main nerve branches through the trunk and into the forelimbs of *Myf-5−/−:MyoD−/−* embryos appeared indistinguishable from that of wild-type embryos; however, no finer motor branches were evident. Limb motor neurons have been suggested to respond to both a general attraction, possibly due to myoblast maturation, and a specific attraction that correlates with target identity (Lance-Jones, 1986; Tosney, 1987). However, in chick wings devoid of muscle, motor neurons project along their normal paths forming normal main nerve trunks and normal cutaneous branches but do not form the finer branches innervating muscle (Lewis *et al.,* 1981; Phelan and Hollyday, 1990). Therefore, our observation that only the main and cutaneous and not the finer motor nerve branches were observed in the limbs of *Myf-5−/−:MyoD−/−* embryos supports the hypothesis that the growth of nerves into the limb is independent of myogenesis and that myocytes are necessary for the formation of finer motor branches. Therefore, the formation of the dorsal ramus appears to be dependent on myogenesis as is the formation of the finer motor branches within the limb bud.

Type Ia afferents in the DRG were observed to undergo apoptosis late in gestation in *Myf-5−/−:MyoD−/−* development. As with *Myf-5−/−:MyoD−/−* embryos, animals

carrying a targeted null mutation in NT-3 are devoid of muscle spindles and display a complete loss of type Ia proprioceptive afferents in the DRG (Ernfors *et al.*, 1994). Proprioceptive sensory neurons are also significantly decreased in mouse embryos where skeletal muscle are eliminated as a consequence of the expression of the gene encoding Diphteria Toxin A-fragment (Grieshammer *et al.*, 1998). However, type Ia afferents form contacts with motor neurons expressing NT-3 at about E17.5 (Ernfors and Persson, 1991), and contacts are formed with muscle spindles several days earlier in development. Therefore, the late elimination of proprioceptive DRG neurons in *Myf-5−/−:MyoD−/−* embryos suggests that it is the formation of active contacts with neurons in the spinal cord rather than formation of contacts with muscle spindles that dictates their survival. However, given that Ia afferents would just be making their initial synaptic connections with motor neurons at E17.5, it is likely that the sensory neurons are also dependent on their peripheral targets which they have contacted much earlier in development.

The TG contains pure motor branches that innervate the masseter muscle, the temporalis muscle, and the pterygoid muscle, but it apparently also contains the primary proprioceptive neurons from the extraocular muscles (Daunicht *et al.*, 1985; Pettorossi *et al.*, 1995). The TG may contain about 18–38 proprioceptive neurons/section and they are either evenly distributed or localized in the medial part of the ganglion in rats (Daunicht *et al.*, 1985). Apoptosis in the examined TG of both wild-type and mutant embryos was evenly distributed in the sections of TG and the observed loss of sensory neurons in the TG in the absence of myogenesis was only 4% greater than in the wild type. In addition, apoptotic elimination of TG neurons occurred earlier (E15.5) than elimination of DRG neurons (E17.5). The early elimination of proprioceptive TG neurons in *Myf-5−/−:MyoD−/−* embryos suggests that it is the formation of active contacts with muscle spindles rather than formation of contacts with neurons in the brain stem that dictates their survival. Therefore, although the difference of 4% was not found to be statistically significant, it could still be evidence of the existence of a very small number of proprioceptive sensory neurons in the TG that underwent muscle-dependent programmed cell death.

The mutant FMN at E17.5 contained small numbers of motor neurons, whereas the thoracic spinal cord contained no somatic motor neurons. We interpret this observation to suggest that motor neuron loss was still in progress in the mutant FMN at E17.5. This finding supports the hypothesis that the primary and secondary muscle fiber requirement for coordinate development of target innervation and motor neuron survival of the FMN may differ from that of the somatic motor neurons in the spinal cord (Brennan *et al.*, 1996). The coordinate development of target innervation is related to the process of neuromuscular specialization, levels of trophic factors, or motor unit size. The observed difference in survival of motor neurons between FMN and the spinal cord could also be cell autonomous. In support of this hypothesis is the observed intrinsic difference between brachial and lumbar motor neurons in the trophic requirements for their survival during embryonic development (Mettling *et al.*, 1993).

The pyramidal tract is regarded as the pathway for cortical control of voluntary movements. The giant pyramidal cells of the pyramidal tract give rise to thick fibers that are believed to be concerned with the finer isolated movements of the distal parts of the extremities. In the medulla oblongata, the corticonuclear fibers terminate in the cranial nerve nuclei. However, the majority of pyramidal tract fibers continue and form contacts with interneurons in the intermediate zone of the spinal cord (the so-called corticospinal tract). Only a small number form direct synaptic contacts with the motor neurons in the anterior horn of the spinal cord. Marked differentiation of pyramidal cells in layer V of the motor cortex starts early in embryonic development (e.g., 5-month-old human fetus) and is suggested to be dependent on the arrival of associative afferent fibers from the white matter at layer V of the motor cortex (Marin-Padilla, 1970).

The motor cortex of term wild-type fetuses contained numerous pyramidal neurons, whereas *Myf-5−/−:MyoD−/−* fetuses displayed a complete absence of morphologically recognizable giant pyramidal cells. However, the absence of pyramidal neurons in the motor cortex of mutant brains was not correlated with increased levels of apoptosis. Therefore, we interpret this result to suggest that differentiation of pyramidal neurons requires formation of contacts of spinal interneurons with motor neurons that themselves form contacts with skeletal muscle. However, it is not possible to exclude that pyramidal neurons are present but atrophied and therefore undetectable by size criteria. Nevertheless, our morphological findings should indicate a disfunction of the corticospinal tract, since morphological changes of the upper motor neurons found in amiotrophic lateral sclerosis result in atrophy of the corticospinal tract (Cotran, 1994). However, the absence of molecular markers for pyramidal neurons and their progenitors hinders the investigation of this issue.

Our analysis of motor system neurogenesis during the development of *Myf-5−/−:MyoD−/−* embryos has revealed a more intimate relationship with myogenesis than has previously been understood. Our data have suggested that the number and arrangement of motor neurons in the thoracic spinal cord change during development to term in a manner that slightly differs from that described for brachial and lumbar spinal cord (e.g., the formation of VMC at the thoracic level that is not present at the brachial level). In the complete absence of myogenesis, motor neurons are completely eliminated by apoptosis, while in the wild-type embryos the overall number of spinal and brain stem motor neurons generated is finely tuned to their target size by apoptosis. We have also suggested that the formation of the dorsal ramus appears to be dependent on myogenesis as is the formation of the finer motor branches within the limb bud. Moreover, we suggest that type Ia sensory neurons

compete to form contacts with motor neurons, while survival of TG proprioceptive sensory neurons appears to be muscle dependent. We also found that in the absence of myogenesis pyramidal neurons in the motor cortex fail to undergo their normal developmental program. Clearly, future characterization of the central nervous system of *Myf-5−/−:MyoD−/−* embryos with molecular markers that define discrete subclasses of neurons should allow insight into the development of neural pathways involved in motor control.

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